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Effects of Arsenic on Osteoblast Differentiation *in Vitro* and on Bone Mineral Density and Microstructure in Rats

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Running Title: Arsenic retards osteoblast differentiation

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Abstract

Background: Arsenic is a ubiquitous toxic element and known to contaminate drinking water in many countries. Several epidemiological studies have shown that arsenic exposure augments the risk of bone disorders. However, the detailed effect and mechanism of inorganic arsenic on osteoblast differentiation of bone marrow stromal cells and bone loss still remain unclear.

Objectives: We investigated the effect and mechanism of arsenic on osteoblast differentiation *in vitro* and evaluated the bone mineral density (BMD) and bone microstructure in rats at doses relevant to human exposure from drinking water.

Methods: A cell model of rat primary bone marrow stromal cells (BMSCs) and a rat model of long-term exposure with arsenic-contaminated drinking water were used. The bone microstructure and BMD in rats were determined by micro-computed tomography (μ-CT).

Results: Exposure of BMSCs to arsenic trioxide (0.5 and 1 μM) significantly attenuated the osteoblast differentiation. The expressions of runt-related transcription factor-2 (Runx2), bone morphogenetic protein-2, and osteocalcin in BMSCs were inhibited and the phosphorylation of enhanced extracellular signal-regulated kinase (ERK) was increased by arsenic treatment during differentiation. These changed differentiation-related molecules could be reversed by ERK inhibitor PD98059. Exposure of rats to arsenic trioxide (0.05 and 0.5 ppm) in drinking water for 12 weeks obviously altered BMD and microstructure, decreased Runx2 expression, and

increased ERK phosphorylation in bones. In BMSCs isolated from arsenic-treated rats, the osteoblast differentiation was inhibited.

Conclusions: These results suggest that arsenic is capable of inhibiting osteoblast differentiation of BMSCs via an ERK-dependent signaling pathway and increasing bone loss.

Introduction

Environmental arsenic pollution causes a significant global problem for human health. Arsenic in the environment contaminates soil and groundwater and is released to food and drinking water. In certain areas of endemic contamination of arsenic in the world, such as Bangladesh, China, India, Mexico, Romania, Taiwan, and others, arsenic-related disease is prevalent as a result of drinking arsenic-contaminated water (Garelick et al. 2008). Arsenic exposure increases the incidence of various complications or disorders, such as hypertension, cardiovascular disorders, skin lesions, cancer, and spontaneous pregnancy loss (Abhyankar et al. 2012; Bloom et al. 2010; Chen et al. 2009). Greater mortality in areas with high arsenic-contaminated drinking water has been found for males and females with several cancers including bone cancer than in the local reference population (Tsai et al. 1999). Arsenic is known to replace phosphorus and localize in the bone, where it may remain for years. Bone marrow abnormality was observed in a patient with severe arsenic poisoning (Feussner et al. 1979). Some epidemiological studies have shown that arsenic exposure augments the risk of bone disorders (Akbal et al. 2013; Haag et al. 1974; Lever 2002). A recent study indicated that arsenic exposure in men subjects is associated with bone metabolism in which arsenic exposure may be the possible cause of osteopenia (Akbal et al. 2013). Hu and colleagues have shown that short-term exposure of high-dose inorganic arsenic (10 mg/kg/day) to rats through an unusual route of arsenic exposure (intraperitoneal injection),

affects the bone remodeling (Hu et al. 2012). However, the detailed effect and mechanism of arsenic on bone system are still unclear.

Arsenic exposure induces the complex modes of action to disturb the physiological functions (Abhyankar et al. 2012; Bailey et al. 2013). Arsenic stress could lead to activate the cellular and molecular signal transduction in target organs (Qian et al. 2003; Wang et al. 2012). Extracellular signal-regulated kinase (ERK), a member of mitogen-activated protein kinases (MAPK), has been found to contribute in arsenic-induced toxicological responses (Bonati et al. 2006; Ivanov and Hei 2013; Wang et al. 2013). Nevertheless, ERK activation also plays an important role in osteoblast differentiation and osteoclast formation (Lai et al. 2001; Matsushita et al. 2009). ERK can also regulate the expressions of osteoblast differentiation-related signaling molecules, such as runt related transcription factor 2 (Runx2), bone morphogenetic protein-2 (BMP-2), and core-binding factor a1 (Celil and Campbell 2005; Wu et al. 2012). However, the effect of arsenic on ERK signaling during osteoblast differentiation still remains unclear. Here, we hypothesized that low-dose inorganic arsenic disturbs osteoblast differentiation from bone marrow stromal cells (BMSCs) through an ERK signaling pathway and induces bone loss. Our results showed that low-dose inorganic arsenic significantly decreases the osteoblast differentiation from BMSCs via an ERK-dependent pathway *in vitro* and *in vivo*.

Materials and Methods

Animal experiments

The Animal Research Committee of College of Medicine, National Taiwan University, approved and conducted the study in accordance with the guidelines for the care and use of laboratory animals. Total thirty-two male Wistar rats (6-8-week-old) were purchased from BioLASCO (Taipei, Taiwan). Two rats were housed in each standard rat microisolator cage on aspen chip bedding in an animal room maintained at 22 ± 2°C having a 24 h light/dark cycle. The animals were treated humanely and with regard for alleviation of suffering. Rats were fed with standard chow diets (LabDiet #5053) and waters *ad libitum*. Deionized and sterile water was provided by bottle with sipper tube attached at the end. The maximum contaminant level of arsenic in drinking water in Taiwan is 0.01 ppm. *In vivo* experiments, rats were randomly divided into three groups (8 animals per group) and received either 0, 0.05, or 0.5 ppm As_2O_3 (Sigma-Aldrich, St. Louis, MO, USA) in drinking water for 12 weeks. Four animals from the total of 8 per group were sacrificed and the left tibiae removed and BMD analysis performed, while the tibias and femurs from the remaining 4 animals were uses to prepare the BMSCs. After 12 weeks of treatment, left tibiae of hind limbs from 4 animals per group were isolated and fixed in PBS containing 4% paraformaldehyde for 48 h. Following, tibiae BMD analysis was performed by micro-computer tomography (μ-CT) (see below). Other tibiae were decalcified with 10% sodium

EDTA solution at 4^{o}C for 1 month. The samples were then embedded in the paraffin and sectioned to a thickness of 4 μm slides for immunofluorescence staining (see below). Moreover, the tibias and femurs from another 4 animals per group were used to prepare the BMSCs (Liu et al. 2011; see below).

Bone marrow cells

Primary BMSCs were isolated from rats and cultured with or without the differentiation medium as previously described (Celebi et al. 2010). Briefly, BMSCs were prepared by removing tibias and femurs from rats under anesthesia (sodium pentobarbital; Sigma-Aldrich, St. Louis, MO, USA) and flushing the bone marrow cavity with growth medium (α-minimum essential medium (αMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 mg/mL streptomycin; Life Technologies, Carlsbad, California, USA). Cells were then cultured in growth medium at 37^{o}C in a humidified atmosphere of 5% CO_2 in air. After a week of cell expansion, the adherent cells were treated with differentiation inducers (10^{-8} M dexamethasone, 10 μM β-glycerophosphate, and 50 μg/mL ascorbic acid; Sigma-Aldrich, St. Louis, MO, USA) in the medium to induce the osteoblast differentiation.

Cell viability assay

BMSCs (2.5×10^4/well) were seeded in the 24-well plate for 24 h and then refreshed by the growth medium. Cells were treated with or without As_2O_3 (0.5-15 μM) for 24 h or As_2O_3 (0.5 and 1 μM) for 3-18 days. Cell viability was measured by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA) assay.

Alkaline phosphatase (ALP) activity assay

An ALP activity assay kit (*Human*, Gesellschaft, Germany) was used. An assay was performed according to the manufacturer's instruction (http://www.standard.com.tw/standard/t_standard/system_ manager/tw/products/uploadFile/64/En-ap-li(ALKALINE).pdf). Briefly, BMSCs (2.5×10^4/well) were treated with or without As_2O_3 (0.5 and 1 μM) for 7 days under differentiation medium. The medium was changed every 3 days. Cells were harvested by the RIPA buffer and centrifuged at 13000 x g for 30 min. The supernatant was measured the ALP activity and read the absorbance at 420 nm. Each sample was normalized by the protein level.

Calcium measurement

A calcium concentration assay kit (Teco diagnostics, Anaheim, CA, USA) was used to detect calcium concentration in culture medium. The assay was performed according to the manufacturer's instruction (http://www. tecodiagnostics.com/wp-content/uploads/2012/07/C504.CA.pdf). Briefly, BMSCs (5 × 105 cells/plate) were cultured in differentiation medium with or without As2O3 (0.5 and 1 μM) for 14 days. The medium was changed every 3 days. Total collected culture media (50 μL) were mixed with working reagent (o-Cresolphthalein Complexone) and calcium buffer for 2-3 min at room temperature. The absorbance was detected at 570 nm and calculated the concentration by the standard curve.

Mineralized nodule formation assay

Mineralization was detected as previously described (Liu et al. 2011). BMSCs (2.5×10^4/well) were cultured in differentiation medium with or without As_2O_3 (0.5 and 1 μM) for 20 days. The medium was changed every 3 days. Cells were washed by PBS buffer and fixed by ice-cold 75% (v/v) ethanol and then stained with 2% (w/v) Alizarin Red S (Sigma-Aldrich, St. Louis, MO, USA). Finally, the stained cells were eluted by the 10% (w/v) cetylpyridinium chloride (Sigma-Aldrich, St. Louis, MO, USA) and measured the absorbance at 550 nm.

Real time PCR

BMSCs (2×10^5/well) were seeded in the 6-well plate for adaptive time. Every three days, the cells were changed with the differentiation medium. Cells were lysed and extracted the total RNA by a kit (Life Technologies, Carlsbad, California, USA). The relative mRNA expression was determined by real-time quantitative PCR as previously described (Hsu et al. 2013). Briefly, total RNA 0.5-1 μg was used for the reverse transcription of RNA to cDNA using avian myeloblastosis virus reverse transcriptase. Each sample (2 μL cDNA) was tested with real-time SYBR Green PCR reagent (Life Technologies) with specific primers: 18S (Forward: AGTCCCTGCCCTTTGTACACA, Reverse: CGATCCGAGGGCCTCACTA); GAPDH (Forward: TGGCACAGTCAAGGCTGAGA, Reverse: CTTCTGAGTGGCAGTGATGG); BMP2 (Forward: AAGCCATCGAGGAACTTTCAGA, Reverse: TCACAGGAAATTTTGAGCTGGC); Osteocalcin (OCN): (Forward: TCTGACAAAGCCTTCATGTCCA, Reverse: CCTAAACGGTGGTGCCATAGAT). Amplification was performed using an ABI StepOnePlus sequence detection system and a StepOne 2.1 software (Applied Biosystems, Foster City, CA, USA).

Western blotting

The Western blotting was performed as described previously (Wu et al. 2011). The 30-50 μg total proteins were subjected to electrophoresis on 8-10% SDS-polyacrylamide gels. The proteins

were electrophoretically transferred to a polyvinylidene difluoride membrane and blocked with 5% fat-free milk in Tris-buffered saline/Tween-20 (TBST) buffer (20 mM Tris, 150 mM NaCl, 0.01% Tween-20, pH 7.5) for 1 h. The primary antibodies for phosphorylated ERK1/2, ERK1/2, Runx2, and GAPDH (Santa Cruze, Santa Cruz, CA, USA) were used. They were incubated in the BSA-TBST buffer overnight at 4°C. The respective secondary antibodies conjugated to horseradish peroxidase were applied for 1 h, after the membranes were washed in PBS and 0.01% Tween-20. The antibody-reactive bands were identified by enhanced chemiluminescence reagent (Millipore, Billerica, MA, USA) and exposed on Kodak radiographic film. The relative values of protein samples were normalized by the internal control GAPDH.

Micro-computed tomography evaluation of trabecular and cortical bones

Tibiae were isolated and fixed in the 4% paraformaldehyde buffer and assessed the bone mineral density (BMD) by μ-CT scanning as described previously (Takahata et al. 2012). Briefly, bones were scanned using μ-CT (Skyscan 1176, Kontich, Belgium) with isotropic high resolution. Scanning was done at 80 keV and 309 μA with aluminum plus copper filter and the images were collected from isolated tibiae. Quantification of trabecular and cortical bone morphometric indices was performed in the regions of metaphysis and diaphysis in the proximal tibiae, respectively. The trabecular/cortical BMD, trabecular bone volume fraction (BV/TV),

trabecular/cortical thickness, and cortical area were measured and analyzed by scanner software (Skyscan CTAn, v.1.1.7).

Immunofluorescence staining

The 4 μm sections of paraffin embedded tibiae bone were deparaffinized with xylene and washed by 90%, 75%, and 50% alcohol for 5 min each. Sections were then treated with 3% hydrogen peroxide methanol solution for eliminating endogenous peroxidase activity and incubated with protease type XIV (0.5 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) for 10 min. The slides were blocked with 5% goat serum for 1 h for non-specific binding reaction and incubated with antibodies for Runx2 and phosphorylated ERK (1:200; Santa Cruz, Santa Cruz, CA, USA) overnight. Slides were then treated with anti-rabbit or anti-mouse FITC fluorescent secondary antibodies (1:500; Sigma-Aldrich, St. Louis, MO, USA) for 1 h. Finally, Hoechst 33258 (1 μg/mL, Sigma-Aldrich, St. Louis, MO, USA) counter staining was performed.

Statistical analysis

Statistical analyses were performed with SPSS-16.0 software. Data are expressed as means± standard deviation (SD). Data were analyzed for statistical significance using one-way analysis of variance (ANOVA) following by Hols-Sidak post analysis to test for differences between groups. A $p \leq 0.05$ was considered statistically significant.

Results

Low-dose arsenic decreases osteoblastogenesis from BMSCs

As shown in Figure 1A, As_2O_3 (3-15 μM) slightly or markedly decreased the BMSCs viability, but low-dose As_2O_3 (0.5 and 1 μM) did not affect the cell viability during culture for 2-18 days (Figure 1B). ALP was significantly decreased by 1 μM at 5 days and by both 0.5 and 1 μM As_2O_3 at 7 days (Figure 2A). The decrease in calcium absorption by both 0.5 and 1 μM As_2O_3 occurred only after 14 days (Figure 2B). The decrease in osteoblast mineralization by both 0.5 and 1 μM As_2O_3 occurred at both 14 and 20 days (Figure 2C). The mRNA expressions of osteoblastogenic markers BMP-2 and osteocalcin (OCN) were also detected. BMP-2 was decreased by both 0.5 and 1 μM As_2O_3 at 5 days and OCN was decreased by 1 μM As_2O_3 at both 10 and 14 days (Figure 2D). These results indicated that non-toxic low-dose As_2O_3 is capable of attenuating the osteoblast differentiation from BMSCs.

ERK signaling plays a role in arsenic-inhibited osteoblastogenesis

ERK phosphorylation has been shown to be involved in the osteoblast differentiation (Ghosh-Choudhury et al. 2007; Lai et al. 2001). We next investigated the effect of low-dose arsenic on the ERK signaling during BMSCs differentiation. As_2O_3 (0.5 and 1 μM) obviously enhanced the ERK phosphorylation during osteoblast differentiation, which could be reversed by ERK inhibitor PD98059 (Figure 3A and 3B). PD98059 also reversed As_2O_3-inhibited Runx2

protein expression (Figure 3B), ALP activity (Figure 3C), osteoblast mineralization (Figure 3D), and BMP-2 (Figure 4A) and osteocalcin (Figure 4B) mRNA expressions during osteoblast differentiation. PD98059 (10 and 20 μM) alone did not affect these osteoblastogenesis markers (Figures 3B-D and 4A-C). These results suggested that As_2O_3 inhibits osteoblast differentiation via an ERK-dependent signaling pathway.

Arsenic alters the bone microstructure and osteoblast differentiation in rats

The body weights in rats 12 weeks after exposure to 0.05 and 0.5 ppm As_2O_3 in drinking water were not significantly affected (control: 334.3 ± 21.5, 0.05 ppm: 339.9 ± 19.2, 0.5 ppm: 345.5 ± 5.0 g; n = 8/group). In As_2O_3-treated rats, bone microstructures of trabecular and cortical bone were altered (Figure 5A). BMD, trabecular bone volume (%BV/TV), and thickness in trabecular bone (Figure 5B), and BMD, thickness, and cortical area in cortical bone (Figure 5C) were significantly decreased. Immunofluorescence staining in bones of As_2O_3-treated rats displayed a decreased Runx2 staining and an increased phosphorylated ERK staining (Figure 6). Furthermore, osteoblast differentiation (Figure 7A) and mineralization (Figure 7B) of BMSCs isolated from bones of As_2O_3-treated rats were significantly decreased. In addition, the ALP activities of BMSCs isolated from As_2O_3-treated rats were also significantly decreased (fold: 0.82 ± 0.09 in 0.05 ppm group and 0.71 ± 0.08 in 0.5 ppm group as compared with control, n = 4,

$p < 0.05$). These results indicated that arsenic exposure causes the inhibition in osteoblast differentiation and alters the bone microstructure and BMD in rats.

Discussion

The main source of arsenic exposure is the arsenic-contaminated drinking water. It has been estimated that arsenic levels in the contaminated areas are several times higher than the maximum contamination level (10 μg/L, 0.01 ppm) (Singh et al. 2007). In West Bengal, about 6 million people have been suspected to be exposed to contaminated drinking water with above the 50 μg/L (0.05 ppm) arsenic concentration (Centeno et al. 2007). A previous epidemiological study in Antofagasta, Chile, has shown that arsenic-related health problems are caused by exposure of contaminated drinking water with arsenic concentrations as high as 800 μg/L (0.8 ppm) (Borgono and Greiber 1971). Arsenic has also been reported to be associated with an increase in the liver cancer mortality in both genders, when arsenic levels are more than 0.64 mg/L (0.64 ppm) (Lin et al. 2013). It has been shown that As_2O_3 induces partial differentiation in acute promyelocytic leukemia cells at low concentrations (0.1-0.5 μM; about 0.02-0.1 ppm), while induces apoptosis at relatively high concentrations (0.5-2 μM; about 0.1-0.4 ppm) (Chen et al. 1997). Similarly, low-dose As_2O_3 (0.1-0.5 μM; about 0.02-0.1 ppm) dose-dependently inhibited *in vitro* skeletal muscle cell differentiation, while induces apoptosis at higher

concentrations (1-10 μM; about 0.2-2 ppm) (Yen et al. 2010; 2012). In addition, significant genetic damage was observed in mice upon exposure to arsenic with human equivalent reference dose (0.3 μg/kg/day) as well as its multiples (1.5-30 μg/kg/day) (Kesari et al. 2012). Arsenic (0.05 and 5 ppm) exposure to mice for 180 days produced obvious DNA damage in bone marrow cells (Singh et al. 2010). Exposure to 2.5-5 μM (about 0.5-1 ppm) arsenite could enhance the differentiation of preosteoclastic cells, suggesting that arsenic may result in increased bone resorption (Szymczyk et al. 2006). Rats treated with arsenite (0.21 mg/kg/day) for 45 days have also been found to increase the thickness of the growth cartilage and the hypertrophic zone, and trabeculae sealed to the cartilage (Odstrcil Adel et al. 2010). Recently, Hu and colleagues have shown that inorganic arsenic at relatively high concentrations (≥ 2 μM; about 0.4 ppm) *in vitro* significantly decrease the differentiation of rat calvaria pre-osteoblasts; furthermore, short-term high-dose arsenic (10 mg/kg/day for 4 weeks) administered by intraperitoneal injection, an unusual route of arsenic exposure, decreased both femur BMD and trabecular bone volume in rats (Hu et al. 2012). Our present study found that submicromolar As_2O_3 (0.5 and 1 μM) significantly reduces the osteoblast differentiation of BMSCs *in vitro*. We further demonstrated that long-term exposure of As_2O_3 in drinking water (0.05 and 0.5 ppm, 12 weeks) to rats, at doses found in drinking water of human arsenic-contaminated areas, significantly decreases the BMD, bone Runx2 expression, and osteoblast differentiation of BMSCs, and increased bone

ERK phosphorylation. These results suggest that exposure to arsenic at doses relevant to human exposure from drinking water may damage the osteoblast differentiation of bone marrow cells and induce the bone loss.

Arsenic can exist in several valency states (-3, 0, +3, and +5) in the environment. It is mostly found in inorganic form as trivalent arsenite (As^{3+}) and pentavalent arsenate (As^{5+}) in natural water. The As^{3+}/As^{5+} ratios in water can greatly vary. In reducing As-rich groundwater from Bangladesh, the ratios of As^{3+} to total arsenic are about 0.1 to 0.9 but are typically around 0.5 to 0.6 (Jiang et al. 2013). A previous study has shown that the kinetic of oxygenation of As^{3+} is slow in the slightly acid range, around pH 5, and it is stable in the anoxic solution for up to 3 weeks (Smedley and Kinniburgh 2002). As_2O_3, a trivalent arsenic compound, can be released into the air and water by natural or industrial processes. As_2O_3 can form arsenite in alkaline solution. In this study, to prevent or minimize oxidation of As_2O_3, the cell culture medium and rat's drinking water containing As_2O_3 were freshly prepared every 2 and 3 days, respectively.

The ERK signaling pathway is known to be involved in cell-matrix interactions in bone and the process of osteoblast differentiation (Ghosh-Choudhury et al. 2007; Lai et al. 2001; Wirries et al. 2013). Wu and colleagues have suggested that the osteoblastic differentiation of bone mesenchymal stem cells is regulated by an ERK-related pathway (Wu et al. 2012). Exposure to

arsenic has been shown to elevate the ERK phosphorylation in various kinds of cells, such as endothelial cells (Wang et al. 2012), keratinocytes (Phillips et al. 2013), and neuronal mesencephalic cells (Felix et al. 2005), protecting against the arsenic-induced damage. On the contrary, a recent study has shown that sodium arsenite diminishes neuronal stem cell differentiation via an over-activation of ERK signaling pathway (Ivanov and Hei 2013). Activation of ERK signaling has also been shown to be involved in the inhibition of osteoblastic differentiation of vascular smooth muscle cells by Ghrelin (Liang et al. 2012) or Taurine (Liao et al. 2008). Tang et al. (2008) reported that PD98059 (20 μM) did not decrease ALP activity in rat osteoblasts. Lin et al. have shown that 20 μM PD98059 potentially induces rat preosteoblst differentiation (Lin et al. 2011). Bai et al. (2013) have reported that 10 μM PD98059 decreased osteoblast differentiation in rabbit BMSCs. In the present study, we found that As_2O_3 activates ERK activation during osteoblast differentiation of BMSCs and ERK inhibitor PD98059 significantly reverses the As_2O_3-inhibited osteoblast differentiation, indicating that arsenic inhibits osteoblastogenesis through an ERK-dependent signaling pathway. Taken together, these previous findings (Tang et al. 2008; Lin et al. 2011; Bai et al. 2013) and our results suggest that ERK activation can lead either to stimulation or to inhibition of osteoblast differentiation pathways, depending on the different system.

Runx2 is a master transcription factor to regulate bone formation and subsequently form the fully functional osteoblasts (Lee et al. 2000). It has been shown that Runx2 activation is regulated by an ERK-dependent signaling pathway in human mesenchymal stem cells (Celil and Campbell 2005). Moreover, the nuclear factor E2 p45-related factor 2 (Nrf2), a transcription factor for the regulation of many detoxifying and antioxidative genes, is known to be activated by ERK signaling (Cai et al. 2012; Khan et al. 2011). Hinoi and colleagues have suggested that Nrf2 can negatively regulate the osteoblast differentiation via an inhibition of the Runx2-dependent transcriptional activity (Hinoi et al. 2006). In the present study, we found that As_2O_3 activates ERK phosphorylation and inhibits Runx2 expression during osteoblast differentiation, which could be reversed by ERK inhibitor. The immunofluorescence co-localization of Runx2 and phosphorylated ERK has been shown in osteoblast cells (Li et al., 2010). The immunofluorescence for Runx2 or phosphorylated ERK in bones (Figure 6) might be mainly localized in osteoblast cells. This arsenic-activated ERK down-regulated Runx2 expression during osteoblast differentiation of BMSCs may be through an ERK-activated Nrf2 signaling pathway. However, the role of Nrf2 in arsenic-inhibited osteoblast differentiation of BMSCs is still needed to be clarified in the future.

Conclusions

In this study, we found that low-dose arsenic significantly reduces the osteoblast differentiation of bone marrow cells *in vitro*. We further demonstrated that long-term exposure of arsenic in drinking water to rats at doses relevant to human exposure from drinking water significantly altered the bone microstructure and BMD. The further evidence from the up-regulation of ERK and inhibitory effect of ERK inhibitor indicated that arsenic inhibits osteoblastogenesis through an ERK-dependent signaling pathway. Taken together, these *in vitro* and *in vivo* findings suggest that inorganic arsenic may be an environmental risk factor for osteoporosis.

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Figure Legends

Figure 1. The effects of As_2O_3 on the cell viability in primary bone marrow stromal cells (BMSCs). BMSCs were isolated from rat bones and cultured with or without As_2O_3 (0.5-15 μM) in growth medium for 48 h (A) or As_2O_3 (0.5 and 1 μM) in differentiation medium for 3-18 days (B). Cell viability was determined by the MTT assay. Data are presented as means ±SD for three independent experiments. *: $P < 0.05$, control group vs As_2O_3 group.

Figure 2. As_2O_3 reduces osteoblast differentiation of BMSCs. BMSCs were cultured in differentiation medium with or without As_2O_3 (0.5 and 1.0 μM) for 5-20 days. ALP activity (A), calcium absorption (B), and osteoblast mineralization (C) during differentiation were measured. Data are presented as means ± SD for three independent experiments. *: $P < 0.05$, control group vs As_2O_3 group. Moreover, the mRNA expressions of BMP-2 and osteocalcin (OCN) were detected by the real-time PCR (D). Results shown are representative of at least three independent experiments. DM: differentiation medium.

Figure 3. As_2O_3 enhances ERK phosphorylation during osteoblast differentiation. BMSCs were cultured in differentiation medium with or without As_2O_3 (0.5 and 1.0 μM) in the presence or absence of ERK inhibitor PD98059 (20 μM) for 6 h to 20 days. ERK phosphorylation (A) and Runx2 expression (B) were determined by the Western blotting. The ALP activity (C) and

osteoblast mineralization (D) were also measured. Data are presented as means ±SD for four independent experiments. *: $P < 0.05$, control group vs As_2O_3 group. #: $P < 0.05$, As_2O_3 group vs As_2O_3 + PD98059 group. DM: differentiation medium.

Figure 4. ERK inhibitor reverses As_2O_3-decreased BMP-2 and OCN mRNA expression during osteoblasts differentiation. BMSCs were cultured in differentiation medium with or without As_2O_3 (0.5 and 1.0 μM) in the presence or absence of ERK inhibitor PD98059 (20 μM) for 5 to 14 days. BMP-2 (A) and OCN (B) were determined by the real-time PCR. Data are presented as means ± SD for three independent experiments. *: $P < 0.05$, control group vs As_2O_3 group. #: $P < 0.05$, As_2O_3 group vs As_2O_3 + PD98059 group. In C, the effect of PD98059 (10 and 20 μM) alone on gene expression of BMP-2 and OCN was shown. Results shown are representative of at least three independent experiments. DM: differentiation medium.

Figure 5. Long-term exposure to As_2O_3 decreases the bone microstructure and BMD in rats. Rats were treated with As_2O_3 (0.05 and 0.5 ppm) in drinking water for 12 weeks (4 rats per group). The regions in the metaphysis and diaphysis of the proximal tibiae were scanned by a μ-CT (A). The trabecular and cortical bone morphometric indices were analyzed. The BMD, trabecular bone volume (%BV/TV), cortical area, and thickness in trabecular bone (B) and

cortical bone (C) were analyzed. Data are presented as means ± SD (n = 4) for 3 independent experiments. *: $P < 0.05$, control group vs As_2O_3 group.

Figure 6. The ERK phosphorylation and Runx2 expression in bones of As_2O_3-treated rats. Rats were treated with As_2O_3 (0.05 and 0.5 ppm) in drinking water for 12 weeks. The tissue sections of tibia bones were performed by the immunofluorescence staining with Runx2 (top) and phosphorylated ERK (bottom). Results shown are representative of at least three independent experiments from 4 rats per group.

Figure 7. Osteoblast differentiation of BMSCs isolated from bones of As_2O_3-treated rats. Rats were treated with As_2O_3 (0.05 and 0.5 ppm) in drinking water for 12 weeks. BMSCs were isolated from bones of control or As_2O_3-treated rats (4 rats per group) and cultured in differentiation medium for 20 days. The osteoblast mineralization was detected (A) and the quantification was analyzed (B). Data are presented as means ± SD (n = 4). *: $P < 0.05$, control group vs As_2O_3 group.

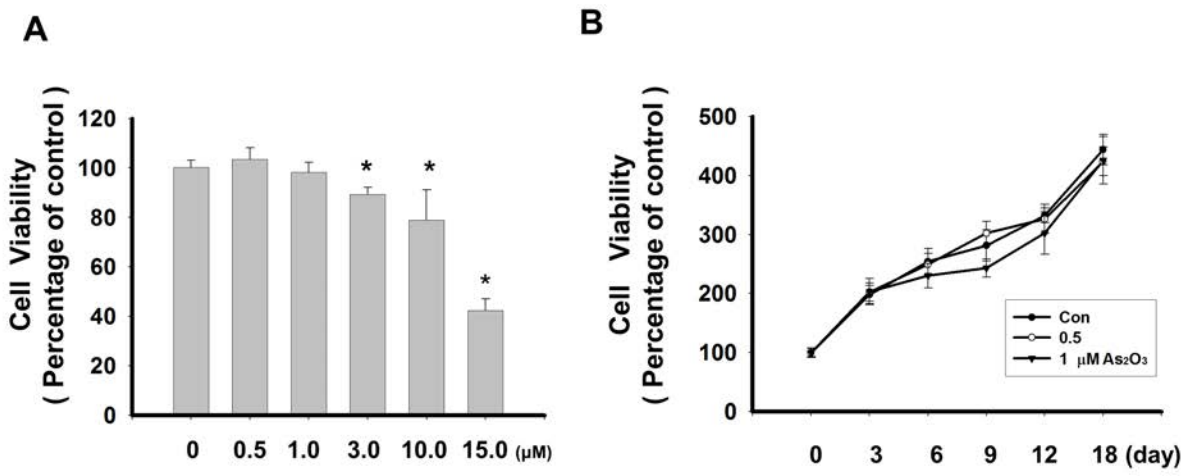
A
Cell Viability
(Percentage of control)
120
100
80
60
40
20
0
*
*
*
0
0.5
1.0
3.0
10.0
15.0 (μM)
B
Cell Viability
(Percentage of control)
500
400
300
200
100
0
Con
0.5
1 μM As2O3
0
3
6
9
12
18 (day)

Fig. 1

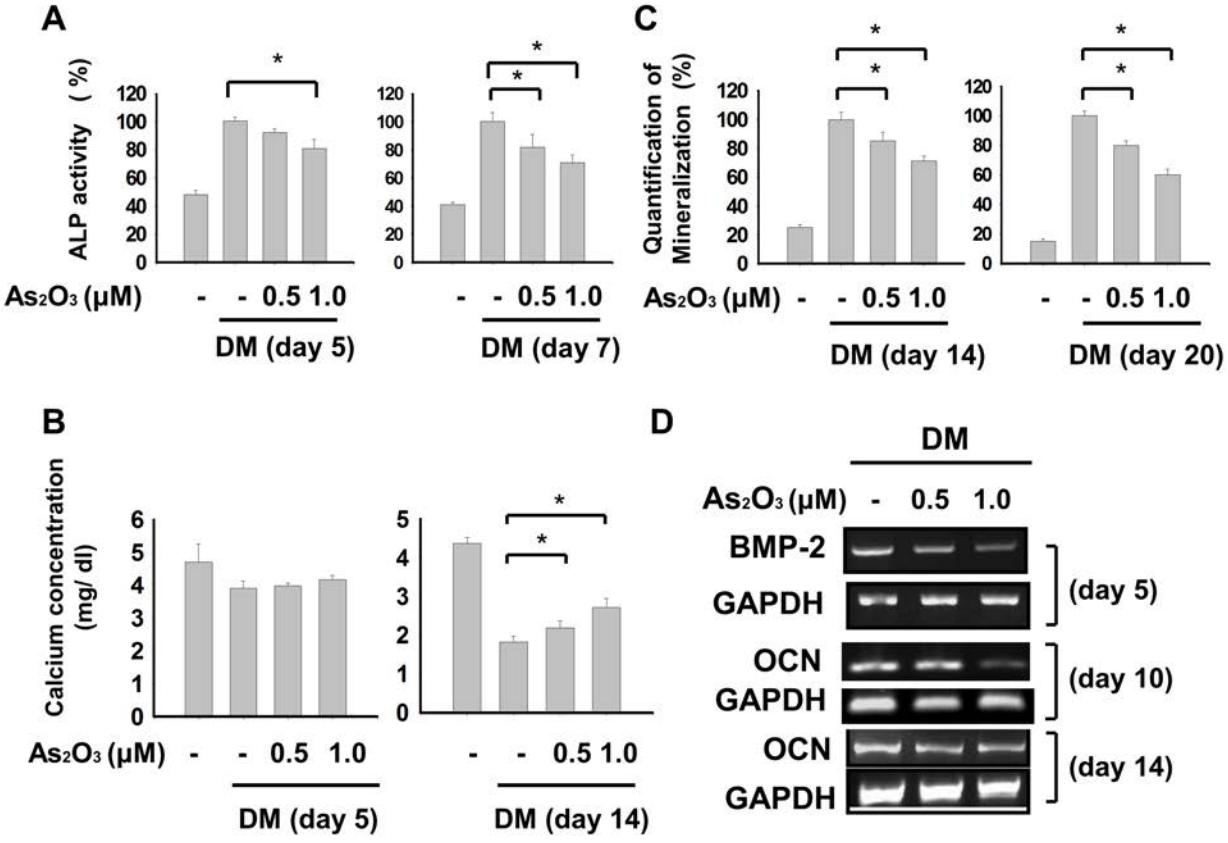
A
ALP activity (%)
120
100
80
60
40
20
0
*
*
*
As2O3 (μM) - - 0.5 1.0
DM (day 5)
As2O3 (μM) - - 0.5 1.0
DM (day 7)
C
Quantification of Mineralization (%)
*
*
*
*
As2O3 (μM) - - 0.5 1.0
DM (day 14)
As2O3 (μM) - - 0.5 1.0
DM (day 20)
B
Calcium concentration (mg/ dl)
6
5
4
3
2
1
0
*
*
As2O3 (μM) - - 0.5 1.0
DM (day 5)
As2O3 (μM) - - 0.5 1.0
DM (day 14)
D
DM
As2O3 (μM) - 0.5 1.0
BMP-2
GAPDH
(day 5)
OCN
GAPDH
(day 10)
OCN
GAPDH
(day 14)

Fig. 2

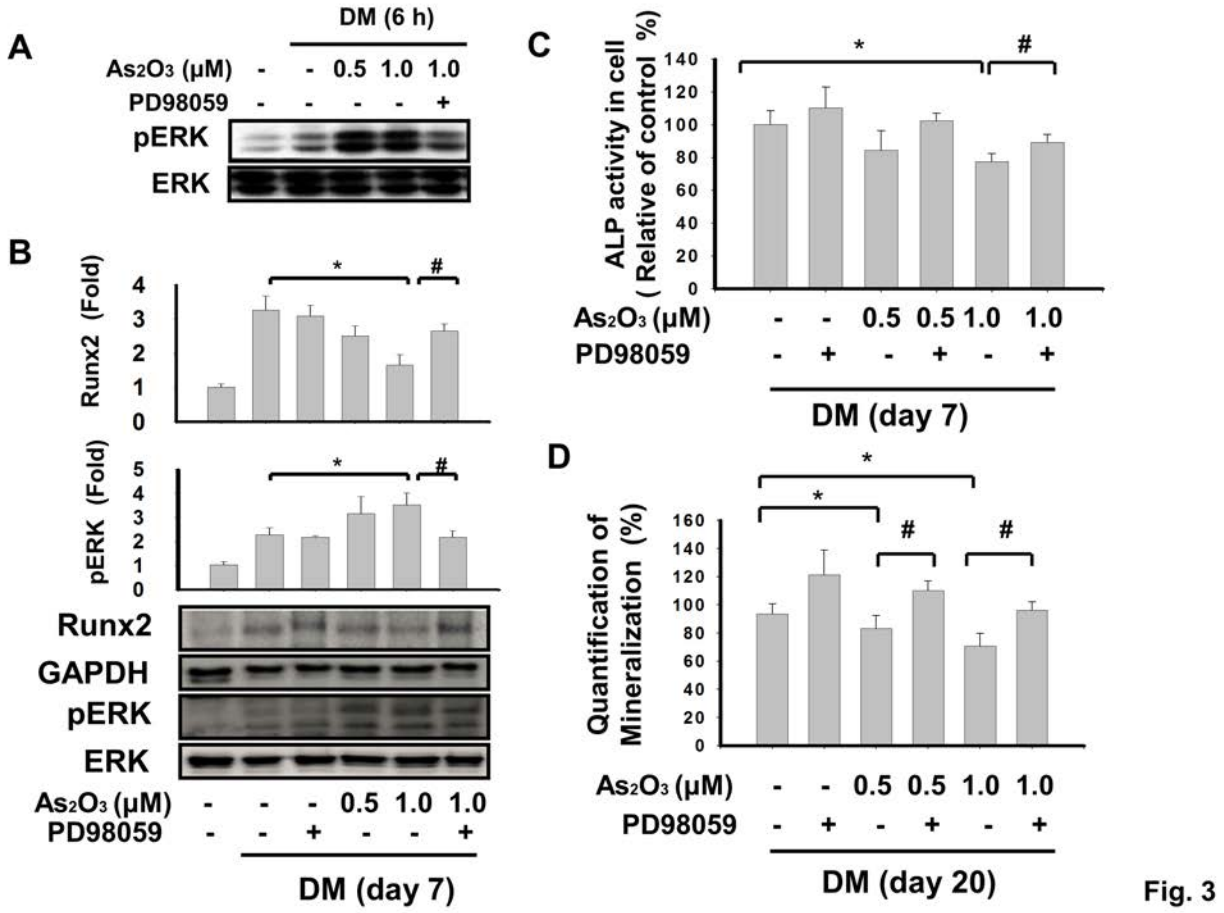
A
DM (6 h)
As2O3 (μM) - - 0.5 1.0 1.0
PD98059 - - - - +
pERK
ERK
B
Runx2 (Fold)
pERK (Fold)
Runx2
GAPDH
pERK
ERK
As2O3 (μM) - - - 0.5 1.0 1.0
PD98059 - - + - - +
DM (day 7)
C
ALP activity in cell (Relative of control %)
As2O3 (μM) - - 0.5 0.5 1.0 1.0
PD98059 - + - + - +
DM (day 7)
D
Quantification of Mineralization (%)
As2O3 (μM) - - 0.5 0.5 1.0 1.0
PD98059 - + - + - +
DM (day 20)

Fig. 3

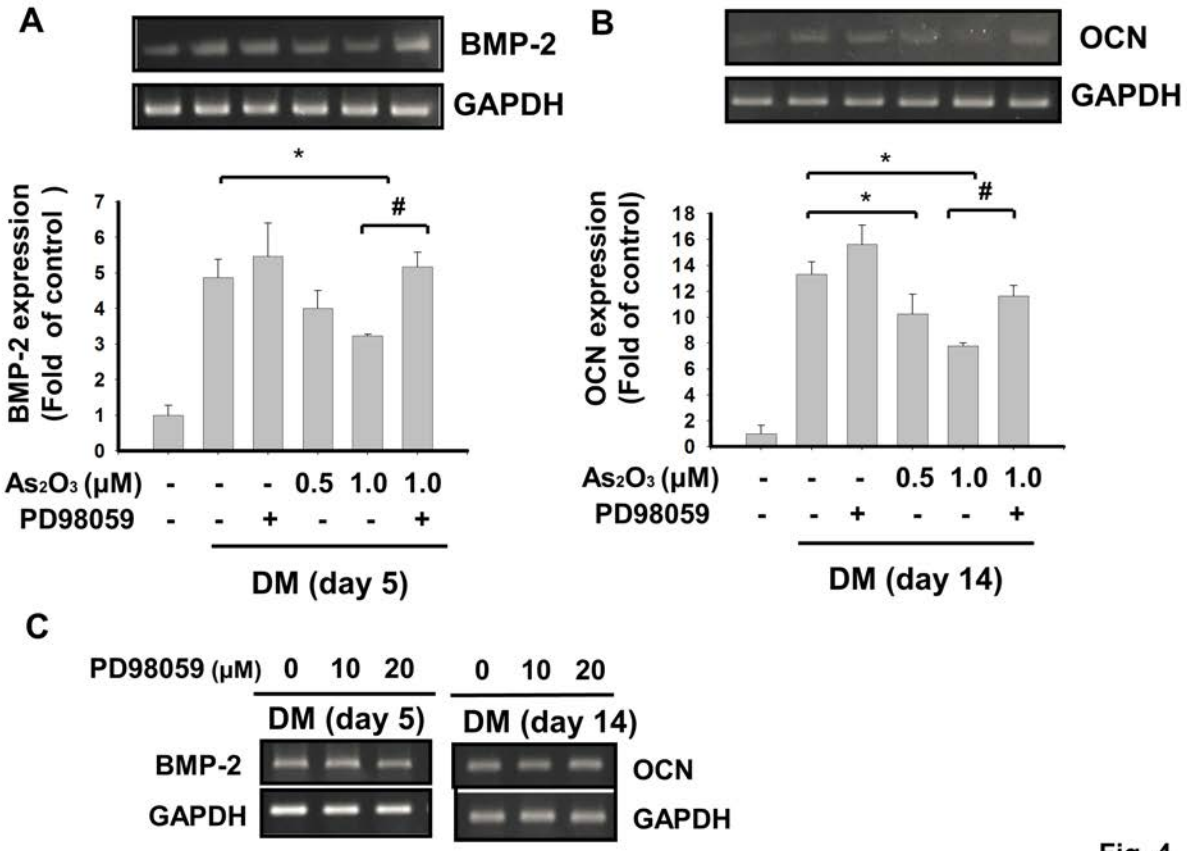
A
BMP-2
GAPDH
BMP-2 expression (Fold of control)
As2O3 (μM) - - - 0.5 1.0 1.0
PD98059 - - + - - +
DM (day 5)
B
OCN
GAPDH
OCN expression (Fold of control)
As2O3 (μM) - - - 0.5 1.0 1.0
PD98059 - - + - - +
DM (day 14)
C
PD98059 (μM) 0 10 20 0 10 20
DM (day 5)
DM (day 14)
BMP-2
GAPDH
OCN
GAPDH

Fig. 4

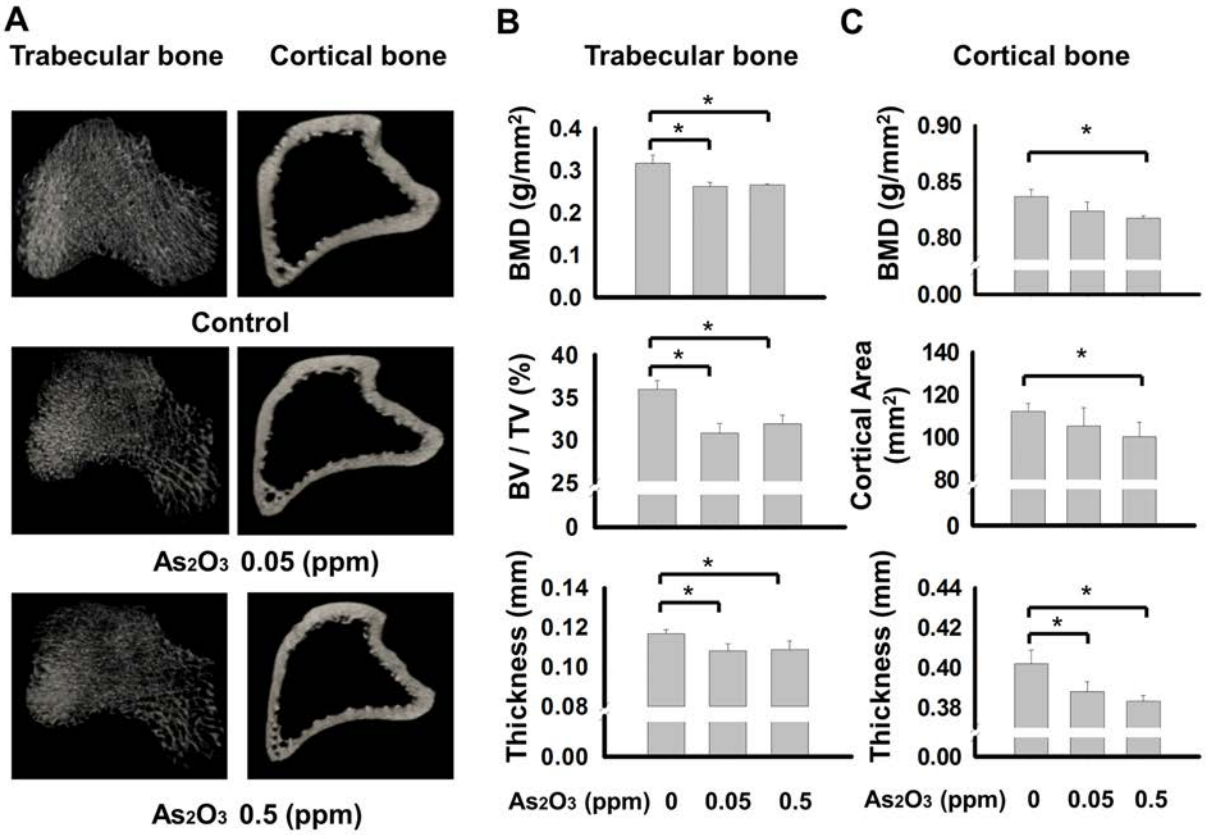
A
Trabecular bone
Cortical bone
Control
As2O3 0.05 (ppm)
As2O3 0.5 (ppm)
B
Trabecular bone
BMD (g/mm2)
BV / TV (%)
Thickness (mm)
As2O3 (ppm) 0 0.05 0.5
C
Cortical bone
BMD (g/mm2)
Cortical Area (mm2)
Thickness (mm)
As2O3 (ppm) 0 0.05 0.5

Fig. 5

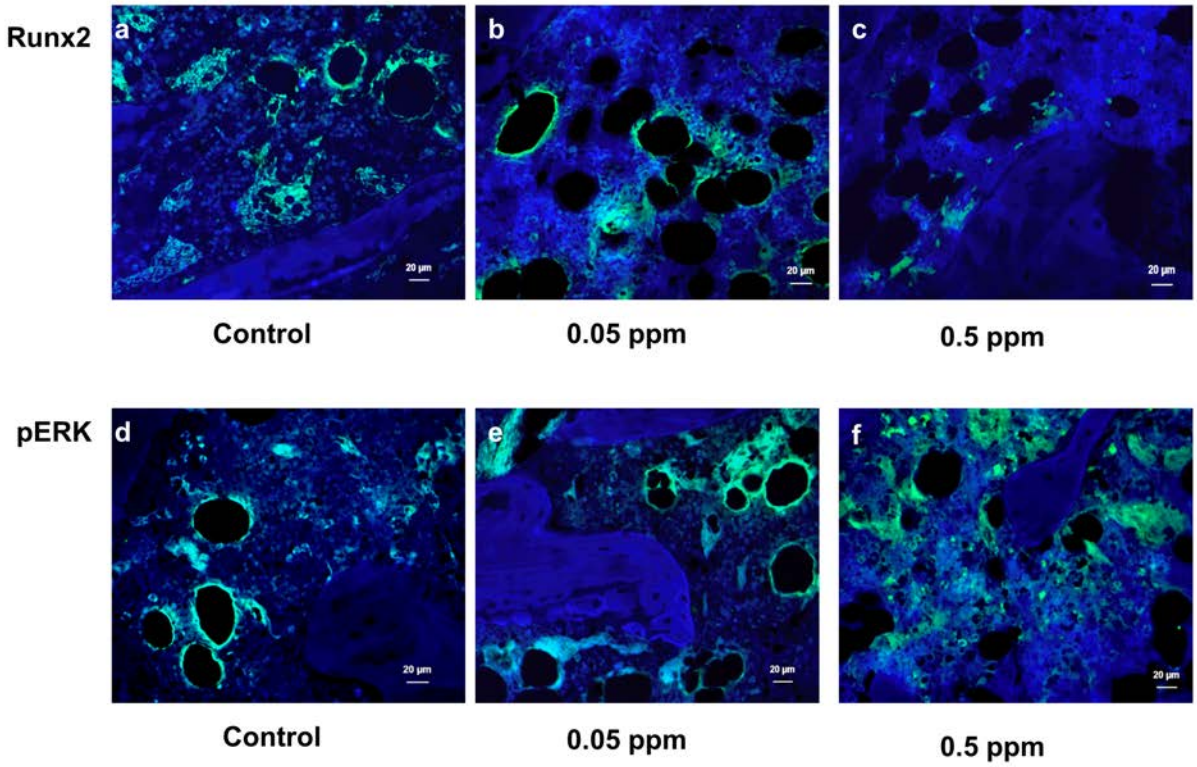
Runx2
a
b
c
20 μm
Control
0.05 ppm
0.5 ppm
pERK
d
e
f
20 μm
Control
0.05 ppm
0.5 ppm

Fig. 6

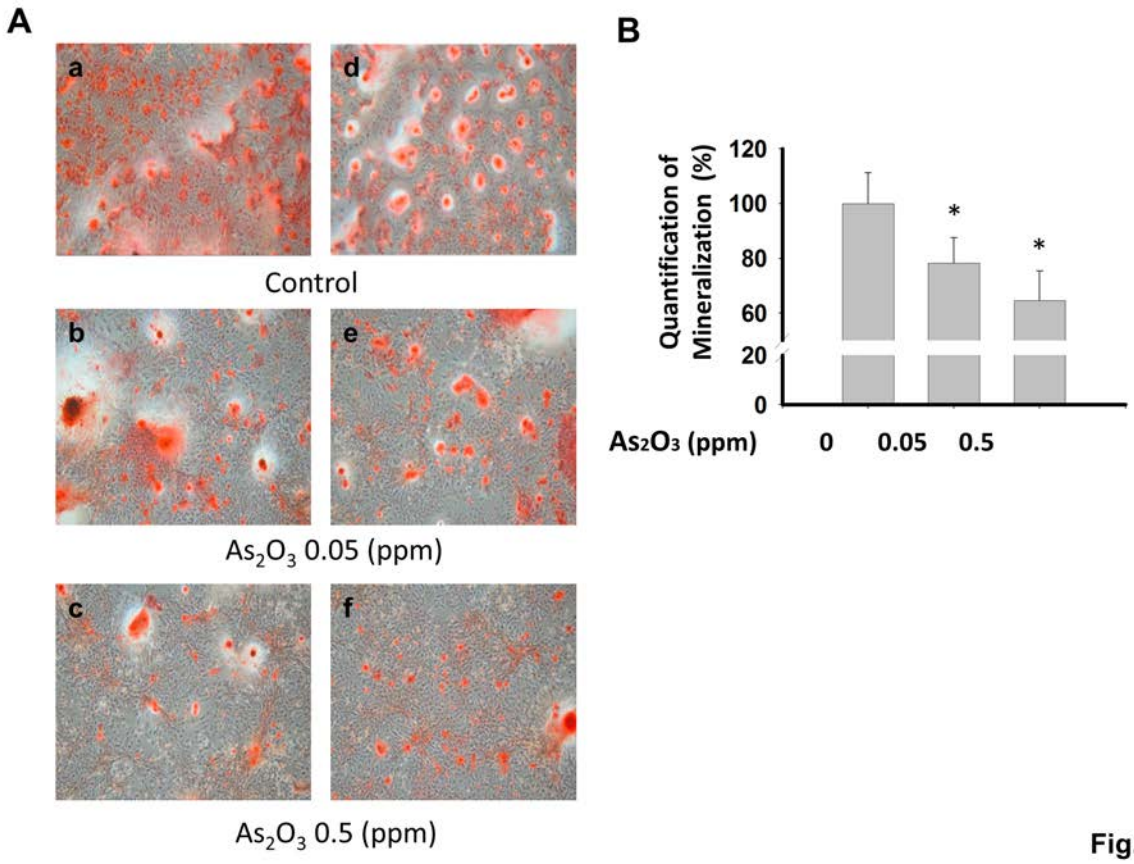
A
a
d
Control
b
e
As2O3 0.05 (ppm)
c
f
As2O3 0.5 (ppm)
B
Quantification of Mineralization (%)
120
100
80
60
20
0
*
*
As2O3 (ppm)
0
0.05
0.5

Fig. 7